

# Regulation of Adenylate Cyclase of Neuroblastoma × Glioma Hybrid Cells by $\alpha$ -Adrenergic Receptors

## I. INHIBITION OF ADENYLATE CYCLASE MEDIATED BY $\alpha$ RECEPTORS\*

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(-)-Norepinephrine and other catecholamines inhibit basal and prostaglandin  $E_1$ -stimulated adenylate cyclase activities by 35 to 60% in homogenates of NG108-15 neuroblastoma × glioma hybrid cells and markedly reduce adenosine 3':5'-monophosphate levels of intact cells, but do not affect guanosine 3':5'-monophosphate levels. The specificity of the NG108-15 receptor for ligands is that of an  $\alpha$  receptor, possibly a presynaptic  $\alpha_2$  receptor. The inhibition of adenylate cyclase by norepinephrine is reversed by  $\alpha$  receptor antagonists such as dihydroergotamine or phentolamine, but not by the  $\beta$  receptor antagonist propranolol. The effect of norepinephrine on adenylate cyclase activity initially is dependent on GTP; half-maximal inhibition of enzyme activity by norepinephrine is obtained with 0.2  $\mu$ M GTP. The inhibition of adenylate cyclase activity by norepinephrine is reduced by 10 mM NaF and is abolished by 0.05 mM guanylyl-5'-yl imidodiphosphate. Inhibitions of NG108-15 adenylate cyclase mediated by  $\alpha$  receptors, opiate receptors, and muscarinic acetylcholine receptors are not additive; this suggests that the three species of receptors can be functionally coupled to the same adenylate cyclase molecules or molecules regulating the enzyme.

Cellular responses to the endogenous catecholamines norepinephrine and epinephrine are mediated by specific receptor molecules, which have been divided into two major classes,  $\alpha$  receptors and  $\beta$  receptors, as well as into subclasses, according to the potencies of ligands for the receptors (reviewed in Ref. 1). Typical  $\alpha$  receptor responses exhibit the specificity, in order of decreasing potency [epinephrine  $\geq$  norepinephrine  $\gg$  isoproterenol], and are blocked by phentolamine, phenoxylbenzamine, or dihydroergotamine. Typical  $\beta$  receptor responses exhibit the specificity [isoproterenol  $>$  epinephrine  $\geq$  norepinephrine] and are blocked by propranolol.

Whereas activation of  $\beta$  receptors usually results in an increase in the activity of adenylate cyclase (EC 4.6.1.1; ATP pyrophosphate-lyase (cyclizing)) and, therefore, an increase in the concentration of adenosine 3':5'-monophosphate (2), the relationship between  $\alpha$  receptor activation and adenylate cyclase is less clearly defined. In certain cells,  $\alpha$  receptor activation reduces the intracellular cAMP concentration or reduces the magnitude of responses elicited by compounds which elevate the cAMP concentration (3-9); thus, Robison *et al.* (2) proposed that  $\alpha$  and  $\beta$  receptors may be linked to

adenylate cyclase in an opposing manner. However, in cerebral cortical slices,  $\alpha$  receptor activation is associated with an increase in cAMP (10-12). In addition,  $\alpha$  receptor activation in certain tissues may increase permeability to  $Ca^{2+}$  ions (13-15) or  $Cl^-$  ions (16) and/or increase cellular cGMP (13, 14) independently of an alteration of cAMP levels.

Clonal NG108-15 hybrid cells,<sup>1</sup> obtained by fusion of mouse neuroblastoma N18TG-2 clone (17) with rat glioma clone C6BU-1 (18), possess adrenergic receptors which, in concert with an activating ligand, reduce the  $PGE_1$ -dependent increase in cellular cAMP (19). The cells also possess  $PGE_1$  and adenosine receptors that are coupled to the activation of adenylate cyclase and opiate and muscarinic acetylcholine receptors coupled to the inhibition of adenylate cyclase (20-23). In this report, we show that  $\alpha$  receptors are coupled to the inhibition of adenylate cyclase in NG108-15 homogenates and characterize the inhibition. In the accompanying report (24), we show that prolonged  $\alpha$  receptor-mediated inhibition of adenylate cyclase in intact NG108-15 cells results in a long lived increase in adenylate cyclase activity. Some of these results have been presented in preliminary form (25). After this work was completed,  $\alpha$  receptor-mediated inhibition of adenylate cyclase in human platelet lysates was reported (26).

### EXPERIMENTAL PROCEDURES

**Growth of Cells and Preparation of Homogenates**—NG108-15 cells (subculture 16-22) were grown in Falcon flasks (75 cm<sup>2</sup> surface area) or Petri dishes (100-mm outside diameter, 64 cm<sup>2</sup> surface area) in 90% DME medium (Grand Island Biological Co., Catalogue No. H-21) containing 44 mM NaHCO<sub>3</sub> and 10% fetal bovine serum (Colorado Serum Co.) supplemented with 0.1 mM hypoxanthine, 1  $\mu$ M aminopterin, and 16  $\mu$ M thymidine in a humidified atmosphere of 90% air, 10% CO<sub>2</sub> at 36.5°C. As cultures approached confluency, the medium was changed once or twice daily to maintain the pH between 7.2 and 7.4. Mycoplasma were not detected in the cells or culture medium.

For preparation of homogenates, cells from confluent cultures (approximately 15 mg of protein/flask) were harvested 4 to 6 h after the medium was replaced with fresh medium. Cells were washed twice with D2 saline solution (0.17 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4, 150 mM NaCl, 5.4 mM KCl, 25 mM D-glucose, and 0.2 mM CaCl<sub>2</sub>) and were dissociated with D1 saline solution (D2 solution without CaCl<sub>2</sub>, pH 6.7) and washed twice with D1 by centrifugation at 250 × *g* for 5 min at 25°C. The final washed cell pellet was suspended in 290 mM

<sup>1</sup> T. Amano, B. Hamprecht, and M. Nirenberg, manuscript in preparation.

<sup>2</sup> The abbreviations used are:  $PGE_1$ , prostaglandin  $E_1$ ;  $PGF_{2\alpha}$ , prostaglandin  $F_{2\alpha}$ ; DME medium, Dulbecco-Vogt modification of Eagle's minimal essential medium; Gpp(NH)p, guanylyl-5'-yl imidodiphosphate; Ro20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)*N,N'*-tetraacetic acid; WB-4101, 2-[(2',6'-dimethoxy)phenoxyethylamino]methylbenzodioxan; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; carbachol, carbamylcholine chloride.

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sucrose, 25 mM Tris-HCl, pH 7.5 (4 to 6 mg of protein/ml), and 1-ml aliquots were frozen in dry ice and stored at  $-190^{\circ}\text{C}$ . Immediately before use an aliquot was thawed and the suspension was homogenized at  $0^{\circ}\text{C}$  in a ground glass tube with 25 strokes of a Teflon pestle rotating at 1200 rpm. The resultant homogenate was found by phase contrast microscopy to have a ratio of intact cells to nuclei of 0.01 or less. Assays for adenylate cyclase activity were initiated within 5 min after homogenates were prepared.

NG108-15 particulate fractions were prepared by centrifugation of homogenates at  $30,000 \times g$  or  $130,000 \times g$ , as indicated in the figure legends, for 15 min. The pellets were washed by dispersion and centrifugation in 290 mM sucrose, 25 mM Tris-HCl, pH 7.5. The final pellets were dispersed in the same buffer (2 mg of protein/ml) and stored at  $-190^{\circ}\text{C}$ . These procedures were performed rapidly because  $\alpha$  receptor-mediated inhibition of adenylate cyclase was found to decay significantly upon storage of homogenates at  $0^{\circ}\text{C}$  for more than 30 min.

Protein was measured by the method of Lowry *et al.* (27) using bovine serum albumin as a standard.

**Assay of Adenylate Cyclase Activity**—Adenylate cyclase activity was routinely determined in 100- $\mu\text{l}$  reaction mixtures containing 30 or 35 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 58 or 116 mM sucrose, 0.25 mM Ro20-1724 (a phosphodiesterase inhibitor), 1 mM [ $\alpha$ - $^{32}\text{P}$ ]ATP (3 to  $6 \times 10^6$  cpm), 1 mM cAMP, 0.1 mM pargyline hydrochloride to inhibit monoamine oxidase, 20 mM creatine phosphate, 10 units (67 to 80  $\mu\text{g}$  of protein) of creatine phosphokinase, 10  $\mu\text{M}$  PGE<sub>1</sub> where indicated, 0.48% ethanol (used as a solvent for PGE<sub>1</sub> and Ro20-1724), and unfractionated NG108-15 homogenate protein as indicated (usually 75 to 110  $\mu\text{g}$  of protein/reaction mixture). Unless otherwise indicated, for assays involving catecholamines, reaction mixtures also contained 0.01 mM sodium ascorbate (see preparation of catecholamine stock solutions below). In most experiments sodium ascorbate had little or no effect on adenylate cyclase activity or on the potency of catecholamines as inhibitors of adenylate cyclase. However, for some homogenates prepared during the later stages of the work, ascorbate was found to activate adenylate cyclase. In these cases, indicated in the text, ascorbate was omitted.

Reactions were started by addition of homogenate to prewarmed (2 min,  $37^{\circ}\text{C}$ ) reaction mixtures. Unless otherwise stated, incubations were at  $37^{\circ}\text{C}$  for 10 min for basal activity and 5 min for PGE<sub>1</sub>-stimulated activity. Reactions were stopped by the addition of 0.8 ml of 6.25% trichloroacetic acid at  $4^{\circ}\text{C}$  and 100  $\mu\text{l}$  of [ $^3\text{H}$ ]cAMP (10,000 cpm, 39.8 Ci/mmol), and cAMP was purified according to method C of Salomon *et al.* (28) with a recovery of [ $^3\text{H}$ ]cAMP of 70 to 85%. Greater than 90% of the  $^{32}\text{P}$  product recovered was shown previously to be cAMP (20). Duplicates usually differed by less than 3%. Less than 0.8% of the 1 mM cAMP in the reaction mixture was degraded by endogenous phosphodiesterase activity in a typical reaction mixture incubated for 10 min.

**Determination of  $\text{IC}_{50}$  and Apparent Hill Coefficient Values**—Adenylate cyclase was determined in the presence of 8 to 12 concentrations of each adrenergic compound tested. The data were fitted to a four-parameter logistic model (29) using the equation:

$$Y = \frac{A - D}{1 + (X/C)^B} + D$$

where  $A$  is the activity in the absence of ligand,  $C$  is the concentration of ligand resulting in half-maximal inhibition ( $\text{IC}_{50}$ ),  $D$  is the maximally inhibited activity at saturating ligand concentration, *e.g.* at least 0.1 mM (–)-norepinephrine,  $B$  is the exponent which is related to the steepness of the dose-response curve and here is termed “apparent Hill coefficient,”  $X$  is the ligand concentration, and  $Y$  is the adenylate cyclase activity. The best values of  $B$  and  $C$  for each concentration curve with given  $A$  and  $D$  values were determined by iterative nonlinear least squares analysis using the MLAB program of the PDP-10 computer of the National Institutes of Health Computer Center. The  $\text{IC}_{50}$  values obtained in this manner agreed well with those obtained by log-probit analysis as well as by inspection of the concentration curves.

**Assay of cAMP of Intact Cells**—Culture medium was removed and 3 ml of cold 5% trichloroacetic acid were added to the cell monolayers. Cells were washed twice with 5% trichloroacetic acid (1 ml/wash). The trichloroacetic acid extracts and washes were pooled and centrifuged at  $30,000 \times g$  for 20 min, and cAMP was purified (20) and assayed by the method of Gilman (30) in 0.2-ml reaction mixtures each containing 1 pmol of [ $^3\text{H}$ ]cAMP and 0.7  $\mu\text{g}$  of partially purified cAMP-dependent protein kinase protein, which bound 0.3 pmol of

[ $^3\text{H}$ ]cAMP under the conditions used. Cell protein was determined (27) from the NaOH-solubilized trichloroacetic acid precipitates.

**Assay of cGMP of Intact Cells**—Confluent cultures of NG108-15 cells in 100-mm Petri dishes were incubated in air for 30 min at  $37^{\circ}\text{C}$  in DME medium containing 25 mM Hepes buffer instead of  $\text{NaHCO}_3$ , adjusted to pH 7.4, and adjusted to 340 mosm/kg with NaCl, and supplemented with 0.1 mM hypoxanthine, 1  $\mu\text{M}$  aminopterin, 16  $\mu\text{M}$  thymidine, and 0.5 mM 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor). Test compounds were then added and dishes were incubated at  $37^{\circ}\text{C}$  for 15 min. The medium was discarded and cells were suspended in 5 ml of 5% trichloroacetic acid. Intracellular cGMP was purified from the trichloroacetic acid supernatants and assayed by radioimmunoassay according to the method of Matsuzawa and Nirenberg (31). The trichloroacetic acid precipitates were assayed for protein (27).

**Chemicals**—The following were kind donations: prostaglandins, Dr. J. E. Pike of Upjohn; Ro20-1724, Dr. H. Sheppard of Hoffmann-La Roche; methoxamine hydrochloride, Burroughs-Wellcome; oxy-metazoline hydrochloride, Schering; phenolamine hydrochloride, CIBA-GEIGY; naloxone hydrochloride, Endo; 9,10-dihydro- $\alpha$ -ergocryptine, Sandoz; fluphenazine hydrochloride, Squibb; and WB-4101, Ward-Blenkinsop Pharmaceuticals.

**R-(–)-Norepinephrine hydrochloride**, **R-(–)-epinephrine bitartrate**, (–)-phenylephrine hydrochloride, (–)-isoproterenol hydrochloride, dopamine hydrochloride, (–)-dopa hydrochloride, 9,10-dihydroergotamine, yohimbine hydrochloride, phenoxybenzamine hydrochloride, (±)-propranolol hydrochloride, atropine sulfate, carbamylcholine chloride, adenosine 5'-triphosphate (prepared from equine muscle or by phosphorylation of adenosine), cAMP-dependent protein kinase (beef heart), and creatine phosphokinase (rabbit muscle) were from Sigma. Pargyline hydrochloride, (±)-dihydroxymandelic acid, and  $\alpha$ -methyl-(±)-norepinephrine were from Regis. Other compounds were from the following sources: (+)-norepinephrine bitartrate, Adams; bulbo-capnine, K and K; morphine sulfate, Merck; guanosine 5'-triphosphate, P-L Laboratories; guanyl-5'-yl imidodiphosphate, ICN; 3-isobutyl-1-methylxanthine, Aldrich; [ $\alpha$ - $^{32}\text{P}$ ]ATP and [ $^3\text{H}$ ]cAMP, New England Nuclear. Other compounds were of reagent grade purity.

Solutions of adrenergic compounds usually were prepared immediately before use and were stored at  $-25^{\circ}\text{C}$  or on ice. Catecholamines were dissolved either in 0.1 mM sodium ascorbate or in 1 mM HCl at  $0^{\circ}\text{C}$ . Stock solutions of dihydroergotamine and dihydroergocryptine were prepared by titrating the free base with HCl (final pH 6.0); these compounds were less soluble at higher pH.

## RESULTS

**Effects of Norepinephrine on cAMP and cGMP Levels of Cells and on Adenylate Cyclase Activity**—The effects of (–)-norepinephrine and PGE<sub>1</sub> on intracellular cAMP concentrations of NG108-15 cells, in the presence or absence of the phosphodiesterase inhibitor Ro20-1724, are shown in Table I (Experiment 1). In the presence of Ro20-1724, 10  $\mu\text{M}$  norepinephrine reduced basal and PGE<sub>1</sub>-stimulated cAMP accumulation by cells to 25%. Cyclic AMP concentrations of cells were low in the absence of the phosphodiesterase inhibitor, and norepinephrine reduced basal cellular cAMP only slightly, if at all, but inhibited PGE<sub>1</sub>-dependent cAMP accumulation by 93%. These results agree well with those reported previously (19, 32). Substitution of 0.2 mM EGTA for  $\text{Ca}^{2+}$  ions in the medium did not significantly reduce the  $\alpha$  receptor-mediated decrease in cAMP accumulation; this suggests that the  $\alpha$  receptor-mediated response is not dependent on extracellular  $\text{Ca}^{2+}$  ions.<sup>3</sup>

The effects of (–)-norepinephrine or PGF<sub>2 $\alpha$</sub>  on intracellular cGMP concentrations of NG108-15 cells are shown in Table I (Experiment 2). Exposure to norepinephrine did not significantly affect the cGMP concentration of cells, but did decrease the cAMP concentration during the period examined. Exposure to PGF<sub>2 $\alpha$</sub>  for 0.5 min, however, elevated the cGMP concentration 5.6-fold as previously found.<sup>4</sup>

<sup>3</sup> R. McGee and M. Nirenberg, unpublished results.

<sup>4</sup> H. Matsuzawa and M. Nirenberg, manuscript in preparation.

As shown in Fig. 1, 10  $\mu$ M norepinephrine inhibited basal and PGE<sub>1</sub>-stimulated adenylate cyclase activities in NG108-15 homogenates 60% and 48%, respectively. Reaction rates were linear during the 15-min period examined. In other experiments (not shown), norepinephrine inhibited adenylate cyclase for at least 60 min; thus, the effect of norepinephrine does not desensitize rapidly under the conditions used. The rates of basal and PGE<sub>1</sub>-stimulated cAMP synthesis were proportional to the amount of homogenate protein added in the range 25 to 150  $\mu$ g of protein/reaction mixture (not shown). The extent of maximum inhibition of adenylate cyclase by norepinephrine varied somewhat from one batch of cells to

another; however, similar values were obtained with separately homogenized portions of the same batch of cells.

**Receptor Specificity for Adrenergic Ligands**—The effects of different ligands and of ligand concentration on basal and PGE<sub>1</sub>-stimulated adenylate cyclase activities are shown in Fig. 2. Compounds known to activate  $\alpha$  receptors, such as (–)-norepinephrine, (–)-epinephrine, and dopamine, inhibited basal and PGE<sub>1</sub>-stimulated adenylate cyclase partially; the maximum inhibitions found were approximately 50 and 35%, respectively. The concentrations of these and other ligands required for half-maximal inhibition of adenylate cyclase (IC<sub>50</sub>) are listed in Table II. Clonidine was the most potent inhibitor tested with an IC<sub>50</sub> of 0.1  $\mu$ M, but the extent of inhibition of basal activity (24%) was less than that of other inhibitors. Clonidine also partially antagonized the inhibition by norepinephrine (not shown), which suggests that clonidine acts as a mixed agonist-antagonist. The IC<sub>50</sub> values for  $\alpha$ -methyl-(±)-norepinephrine, (–)-norepinephrine, and (–)-epinephrine were 0.2, 0.4, and 0.5  $\mu$ M, respectively, for basal activity, while dopamine and (–)-isoproterenol, a selective  $\beta$  receptor activator, were less potent inhibitors with IC<sub>50</sub> values of 5 and 60  $\mu$ M, respectively. (+)-Norepinephrine was 75-fold less potent an inhibitor than (–)-norepinephrine; this indicates that the inhibition of adenylate cyclase is dependent on a stereospecific interaction. (–)-Phenylephrine, methoxamine, and oxymetazoline, which are potent  $\alpha$  receptor activators in other systems, were relatively weak inhibitors of adenylate cyclase (IC<sub>50</sub> 9 to 80  $\mu$ M). Serotonin, an  $\alpha$  receptor activator in some systems, did not reduce intracellular cAMP levels<sup>4</sup> or inhibit adenylate cyclase. A precursor of norepinephrine (dopa) and a metabolite (3,4-dihydroxymandelic acid) did not affect adenylate cyclase activity. Thus, the receptor exhibits the specificity of an  $\alpha$  receptor. The potency order [ $\alpha$ -meth-

TABLE I  
Effects of norepinephrine on intracellular cAMP and cGMP of NG108-15 cells

Experiment 1: duplicate Petri dishes (60-mm diameter), each with 3 mg of cell protein, were incubated in an atmosphere of 10% CO<sub>2</sub>, 90% air with 5 ml of growth medium/dish without serum, supplemented with 0.1 mM ascorbic acid and 0.1 mM pargyline, with or without 250  $\mu$ M Ro20-1724, for 20 min at 37°C. Ten micromolar PGE<sub>1</sub> and/or 10  $\mu$ M (–)-norepinephrine was then added where indicated. Dishes were incubated for an additional 10 min at 37°C, then intracellular cAMP was determined. Experiment 2: triplicate Petri dishes (100-mm diameter), each with 10 mg of cell protein, were incubated as described under "Experimental Procedures." Then the following compounds were added where indicated: 1  $\mu$ M HCl (control), 10  $\mu$ M (–)-norepinephrine and 1  $\mu$ M HCl, or 10  $\mu$ M PGF<sub>2 $\alpha$</sub> . Cultures were incubated at 37°C for the times indicated, then intracellular cGMP and, where indicated, cAMP were determined.

Experiment No. and Additions	Min-utes	Picomoles cAMP/mg protein $\pm$ S.E.	Picomoles cGMP/mg protein $\pm$ S.E.
1. cAMP: With Ro20-1724			
None	10	160 $\pm$ 4	
Norepinephrine	10	38 $\pm$ 10	
PGE <sub>1</sub>	10	4100 $\pm$ 490	
PGE <sub>1</sub> + norepinephrine	10	1040 $\pm$ 57	
cAMP: Without phosphodiesterase inhibitor			
None	10	12 $\pm$ 3	
Norepinephrine	10	10 $\pm$ 1	
PGE <sub>1</sub>	10	790 $\pm$ 67	
PGE <sub>1</sub> + norepinephrine	10	52 $\pm$ 11	
2. cGMP			
Control	0		0.57 $\pm$ 0.12
Control	0.5		0.53 $\pm$ 0.09
Control	2.0	27 $\pm$ 2	0.47 $\pm$ 0.03
Norepinephrine	0.25		0.51 $\pm$ 0.04
Norepinephrine	0.5		0.55 $\pm$ 0.15
Norepinephrine	1.0		0.58 $\pm$ 0.16
Norepinephrine	2.0	16 $\pm$ 1	0.36 $\pm$ 0.04
PGF <sub>2<math>\alpha</math></sub>	0.5		3.0 $\pm$ 0.20

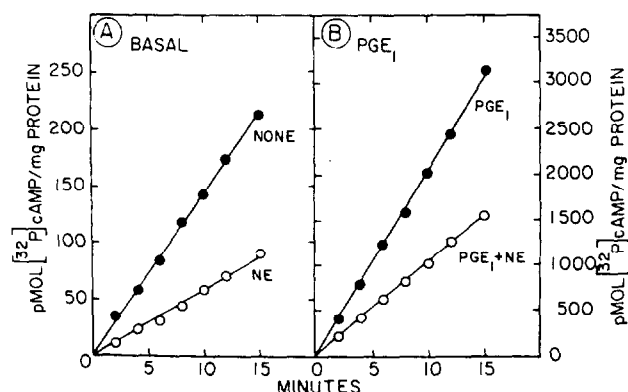


FIG. 1. Effect of norepinephrine (NE) (10  $\mu$ M) on the rate of [<sup>32</sup>P]cAMP formation in an NG108-15 homogenate. Aliquots (50  $\mu$ l) were withdrawn at the indicated times from a 600- $\mu$ l reaction mixture containing 735  $\mu$ g of homogenate protein. A, basal rate; B, rate in the presence of 10  $\mu$ M PGE<sub>1</sub>.

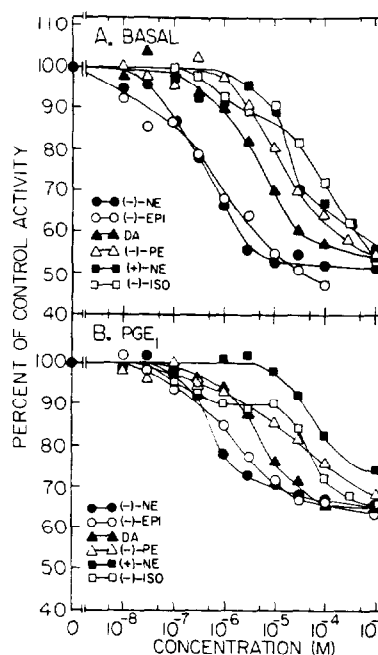


FIG. 2. Inhibition of (A) basal and (B) PGE<sub>1</sub>-stimulated adenylate cyclase activity by adrenergic compounds. Each reaction contained 100  $\mu$ g of homogenate protein and one of the following compounds at the concentrations indicated: (–)-norepinephrine ((–)-NE), (–)-epinephrine bitartrate (EPI, bitartrate concentration adjusted with sodium salt to 0.1 mM in all tubes), dopamine (DA), (–)-phenylephrine (PE), (+)-norepinephrine ((+)-NE), and (–)-isoproterenol (ISO). One hundred per cent corresponds to the following specific activities (picomoles of [<sup>32</sup>P]cAMP/min/mg of protein) for the compounds listed: (A) 11.1, 10.6, 10.4, 10.3, 9.0, 10.5, respectively; and (B) 142, 167, 144, 191, 167, and 155, respectively.

TABLE II

Concentrations of adrenergic compounds required for inhibition of NG108-15 adenylate cyclase activity

Adenylate cyclase activity of homogenates (average 105  $\mu$ g of protein/reaction mixture) was assayed with or without 10  $\mu$ M PGE<sub>1</sub> in the presence of 10 to 12 concentrations of each compound in duplicate reaction mixtures. Half-maximal inhibitions ( $IC_{50} \pm S.E.$ ) were found from the resultant concentration curves as described under "Experimental Procedures." Numbers in parentheses refer to the number of experiments averaged. Otherwise, values listed refer to the most reliable experiment.

Compound	$IC_{50}$	
	Basal	PGE <sub>1</sub>
	$\mu$ M	
Clonidine	0.1 <sup>a</sup>	
$\alpha$ -Methyl-( $\pm$ )-norepinephrine	0.2	0.1
(-)-Norepinephrine	0.4 $\pm$ 0.1 (9)	0.6 $\pm$ 0.2 (2)
(-)-Epinephrine	0.5 $\pm$ 0.03 (3)	0.3
Dopamine	5	20
Oxymetazoline	9	
(-)-Phenylephrine	20	30
(+)-Norepinephrine	30	160
(-)-Isoproterenol	60	30
( $\pm$ )-Methoxamine	80	70
(-)-Dopa	>100 <sup>b</sup>	
(-)-3,4-Dihydroxymandelic acid	>100 <sup>b</sup>	
Serotonin	>100 <sup>b</sup>	>100 <sup>b</sup>

<sup>a</sup> Maximum inhibition less than that for catecholamines.

<sup>b</sup> Little or no effect at 100  $\mu$ M, the highest concentration tested.

yl-norepinephrine > norepinephrine  $\gg$  phenylephrine > methoxamine] suggests that NG108-15  $\alpha$  receptors resemble presynaptic  $\alpha_2$  receptors more than postsynaptic  $\alpha_1$  receptors of smooth muscle (33-35).

Average apparent Hill coefficients obtained from the dependence of inhibition of basal and PGE<sub>1</sub>-stimulated adenylate cyclase on ligand concentration were as follows: (-)-norepinephrine,  $0.71 \pm 0.04$  ( $n = 9$ ) and  $0.91 \pm 0.16$  ( $n = 2$ ), respectively; and (-)-epinephrine,  $0.72 \pm 0.11$  ( $n = 3$ ) and  $0.67 \pm 0.16$  ( $n = 2$ ), respectively. The average apparent Hill coefficient for other ligands was 0.8. These results suggest either heterogeneity of receptors or adenylate cyclase or negative cooperativity in ligand-receptor interactions and/or in the functional coupling of the [ligand-receptor] complex with adenylate cyclase. Therefore, the  $IC_{50}$  values (Table I) do not necessarily approximate dissociation constants for the [ligand-receptor] complexes.

The inhibition of adenylate cyclase by norepinephrine was blocked by the reversible  $\alpha$  receptor antagonists dihydroergotamine and phentolamine as well as the irreversible  $\alpha$  receptor antagonist phenoxybenzamine, but not by the  $\beta$  receptor antagonist propranolol (Fig. 3). The  $\alpha$  antagonist property of phentolamine and phenoxybenzamine could be demonstrated only in the presence of 10 to 50  $\mu$ M naloxone, a specific opiate receptor antagonist. This is because phentolamine or phenoxybenzamine also were weak activators of the opiate receptor and thereby inhibited NG108-15 adenylate cyclase (half-maximal inhibition at 2  $\mu$ M or 5  $\mu$ M, respectively). These compounds are known to interact with rat brain opiate receptors (36).

The apparent dissociation constants ( $K_{Dapp}$ ) of various receptor antagonists in reversing norepinephrine-dependent inhibition of adenylate cyclase are shown in Table III. The specificity of the receptor for antagonists is consistent with that of an  $\alpha$  receptor, with dihydroergocryptine, dihydroergotamine, and yohimbine the most potent tested ( $K_{Dapp}$  0.005 to 0.07  $\mu$ M). The  $K_{Dapp}$  of phentolamine (0.2  $\mu$ M) was higher

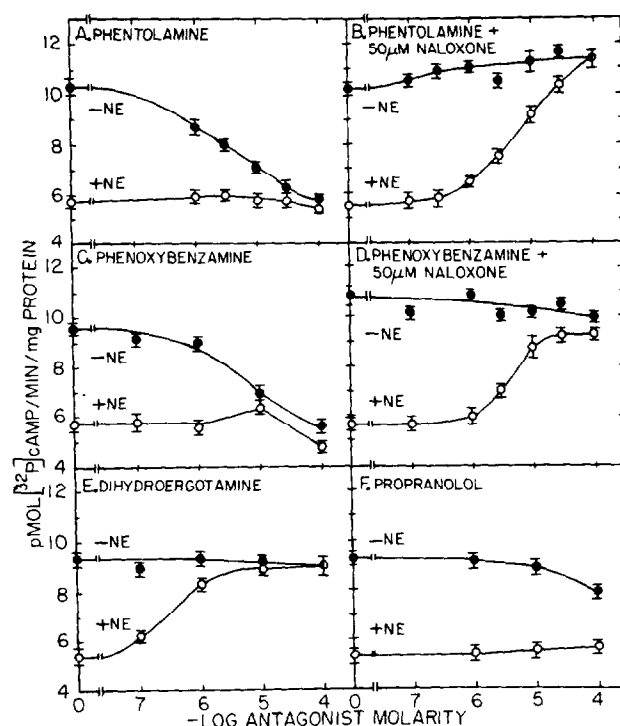


FIG. 3. Effect of adrenergic receptor antagonists on the norepinephrine-dependent inhibition of basal NG108-15 adenylate cyclase. Reaction mixtures without (●) or with (○) 10  $\mu$ M (-)-norepinephrine (NE) contained 108  $\mu$ g of homogenate protein and receptor antagonist at concentrations indicated.

TABLE III

Apparent dissociation constants of receptor antagonists in blocking norepinephrine-dependent inhibition of adenylate cyclase activity

Basal adenylate cyclase activity was assayed with or without 10  $\mu$ M (-)-norepinephrine (NE) in the presence of different concentrations of antagonist. The concentrations ( $EC_{50}$ ) resulting in half-maximal reversal of norepinephrine inhibition were used to calculate the apparent dissociation constants  $K_{Dapp}$  of the antagonist from the equation (37)  $K_{Dapp} = EC_{50}/(1 + [NE]/K_{Sapp})$ , where  $K_{Sapp}$  is the apparent dissociation constant for norepinephrine- $\alpha$  receptor binding, assumed here to be 0.4  $\mu$ M (Table I).

Compound	$K_{Dapp}$ $\mu$ M
9,10-Dihydro- $\alpha$ -ergocryptine <sup>a</sup>	0.005
9,10-Dihydroergotamine <sup>b</sup>	0.01
Yohimbine	0.07
Phentolamine <sup>c</sup>	0.2
Phenoxybenzamine <sup>c,d</sup>	0.2
WB-4101	0.2
Fluphenazine	0.2
( $\pm$ )-Propranolol	>100 <sup>e</sup>
Bulbocapnine	>100 <sup>e</sup>
Atropine	>100 <sup>e</sup>
Naloxone	>100 <sup>e</sup>

<sup>a</sup> Mixed agonist-antagonist effect noted.

<sup>b</sup> Mixed agonist-antagonist effect noted in some but not all experiments.

<sup>c</sup> Naloxone (50  $\mu$ M) added to reaction mixtures to block opiate receptor activation.

<sup>d</sup> Irreversible antagonist.

<sup>e</sup> Little or no effect at 100  $\mu$ M, the highest concentration tested.

than that found in other systems (about 0.01  $\mu$ M), but is identical with that found for the reversal of  $\alpha$ -adrenergic inhibition of platelet adenylate cyclase (38). Fluphenazine, a potent antagonist of dopamine receptors in the central nervous system (39), blocked NG108-15 receptors with a  $K_{Dapp}$  of 0.2  $\mu$ M, which is consistent with the  $\alpha$  antagonist property of

phenothiazines. The receptor antagonists propranolol ( $\beta$  receptors), bulbo-capnine (dopamine receptors), atropine (muscarinic acetylcholine receptors), and naloxone (opiate receptors) had little or no effect on norepinephrine-dependent inhibition of adenylate cyclase at concentrations of 100  $\mu$ M or less.

**Responses of Parent Cell Lines of the Hybrid Line NG108-15**—As shown in Table IV, adenylate cyclase activity in homogenates of C6BU-1 rat glioma cells was stimulated by norepinephrine; however, when the  $\beta$  receptors of these cells were blocked by propranolol, norepinephrine had no effect on adenylate cyclase activity. However, adenylate cyclase of N18TG-2 mouse neuroblastoma cells was inhibited by norepinephrine, but slightly less than that of the hybrid cells. This suggests that expression of  $\alpha$  receptors in the hybrid cells is a property derived from the neuroblastoma parent.

**Effect of GTP**—Hormonal stimulation of adenylate cyclase has been shown to require low concentrations of GTP (see Ref. 40 for review). To determine whether  $\alpha$  receptor-mediated inhibition of NG108-15 basal adenylate cyclase requires GTP, a washed particulate fraction was assayed with or without GTP in a system in which the ATP concentration was reduced to 0.1 mM and ATP synthesized by phosphorylation of adenosine was used to reduce the level of guanine nucleotide contaminants. In the absence of added GTP (Fig. 4A), norepinephrine did not inhibit the initial rate of cAMP synthesis between 0 and 4 min, but norepinephrine-dependent inhibition slowly appeared during further incubation. However, in the presence of 1  $\mu$ M GTP (Panel B), the lag in the initial rate was abolished and norepinephrine inhibited adenylate cyclase activity approximately 45% at each time tested between 2 and 15 min. The relationship between GTP concentration and the reaction rates between 0 and 4 min and between 4 and 8 min are shown in Panels C and D, respectively. Between 0 and 4 min, three effects of GTP on adenylate cyclase can be seen: (a) stimulation of the initial rate (half-maximal stimulation at  $5 \times 10^{-8}$  M GTP); (b) enhancement of norepinephrine-dependent inhibition from 6% to 44% (half-maximal effect at  $3 \times 10^{-7}$  M GTP); and (c) inhibition of activity in the absence or presence of norepinephrine ( $>10^{-6}$  M GTP). Between 4 and 8 min, GTP did not stimulate the rate of cAMP synthesis but increased norepinephrine-dependent inhibition from 17% to 46% (half-maximal effect at  $3 \times 10^{-7}$  M GTP) and inhibited activity above  $10^{-6}$  M GTP. These results indicate that inhibition of adenylate cyclase by norepinephrine is dependent upon GTP.

TABLE IV

Effect of norepinephrine on adenylate cyclase activity of neuroblastoma  $\times$  glioma hybrid NG108-15 cells and parental cell lines

Adenylate cyclase reaction mixtures contained, where indicated, homogenate protein from C6BU-1 cells (subculture 24, 170  $\mu$ g), N18TG-2 (subculture 12, 146  $\mu$ g), or NG108-15 cells (190  $\mu$ g) and 10  $\mu$ M (—)norepinephrine, 10  $\mu$ M PGE<sub>1</sub>, or 20  $\mu$ M ( $\pm$ )propranolol, as indicated. The activities listed are means of duplicate determinations.

Additions	Parental cells		
	Rat glioma C6BU-1	Mouse neuroblastoma N18TG-2	Neuroblastoma $\times$ glioma hybrid NG108-15
	pmol cAMP/min/mg protein		
None	43	5.5	7.7
Norepinephrine	113	4.1	4.6
PGE <sub>1</sub>	48	97	128
PGE <sub>1</sub> + norepinephrine	116	76	94
Propranolol	43		
Propranolol + norepinephrine	43		

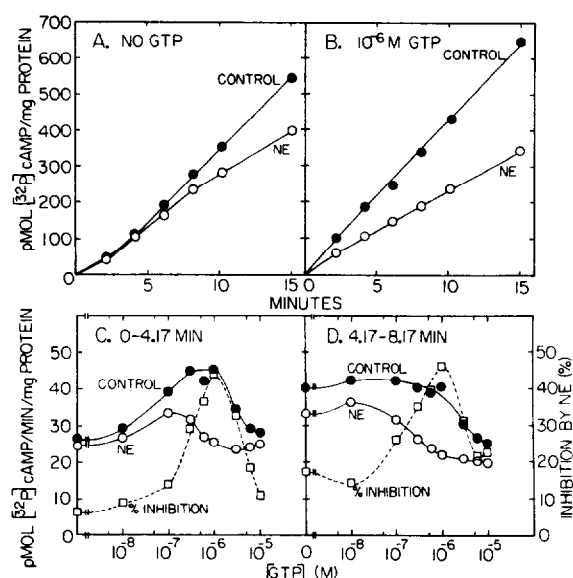


FIG. 4. Effect of GTP on inhibition of adenylate cyclase by norepinephrine. Reaction mixtures (400  $\mu$ l) contained 0.1 mM [ $\alpha$ -<sup>32</sup>P]ATP (unlabeled ATP prepared by phosphorylation of adenosine), 129  $\mu$ g of protein of NG108-15 particulate fraction (30,000  $\times$  g, 15 min), and, as indicated, 100  $\mu$ M norepinephrine (NE) and 0 to 10  $\mu$ M GTP. A and B, adenylate cyclase activity in the absence or presence of 1  $\mu$ M GTP, respectively, as a function of time. C and D, average specific activities between 0 and 4.17 min or 4.17 and 8.17 min, respectively, in the absence or presence of 100  $\mu$ M norepinephrine and per cent inhibition by norepinephrine (dashed lines).

**Effects of Sodium Fluoride and Gpp(NH)p**—Sodium fluoride (reviewed in Ref. 41) and guanylyl-5'-yl imidodiphosphate (42) activate adenylate cyclase from many sources, but the fully activated enzyme then appears to be unresponsive to hormones which usually increase its activity. NaF or Gpp(NH)p activate NG108-15 adenylate cyclase and concomitantly reduce or abolish the responsiveness of the enzyme to inhibition by opiates or activation by PGE<sub>1</sub> (20, 21). As shown in Fig. 5, 10 mM NaF or 50  $\mu$ M Gpp(NH)p increased activity of NG108-15 adenylate cyclase to 3.4 or 2.8 times, respectively, the basal activity. Whereas 10  $\mu$ M norepinephrine decreased the basal activity by 41%, norepinephrine reduced the activity of the fluoride-stimulated enzyme by only 11% and did not reduce the activity of the Gpp(NH)p-stimulated enzyme. These results show that NaF and Gpp(NH)p reduce the effectiveness of norepinephrine as an inhibitor of adenylate cyclase and suggest that these compounds affect either interaction of the [norepinephrine- $\alpha$  receptor] complex with adenylate cyclase and/or the binding of norepinephrine to the receptor.

**Nonadditivity of Adenylate Cyclase Inhibition Mediated by Three Species of Receptors**— $\alpha$  Receptors, opiate receptors, and muscarinic acetylcholine receptors of NG108-15 cells each mediate only a partial inhibition of adenylate cyclase. Thus, the question arises as to whether each species of receptor can become functionally coupled to the same population or to distinct populations of adenylate cyclase molecules. To investigate this possibility, enzyme activity was determined in the presence of activators of each species of receptor (norepinephrine, morphine, and carbachol, respectively), separately, and in various combinations (Table V). Each ligand was tested at a concentration which resulted in at least 90% of the maximum inhibition obtained with a saturating concentration of ligand as determined by the dependence of inhibition on the concentration of norepinephrine (Fig. 2), morphine (20), or carbachol.<sup>5</sup> The maximum inhibitions by norepinephrine or mor-

<sup>5</sup> S. K. Sharma and M. Nirenberg, manuscript in preparation.

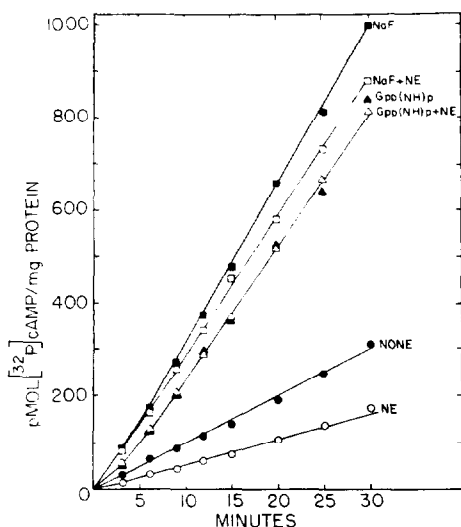


FIG. 5. Effect of 50  $\mu$ M guanylyl-5'-yl imidodiphosphate and 10 mM sodium fluoride on adenylate cyclase inhibition by 10  $\mu$ M (—) norepinephrine (NE). Each reaction mixture (700  $\mu$ l) contained 875  $\mu$ g of NG108-15 homogenate protein. Aliquots of 50  $\mu$ l were withdrawn at indicated times. Symbols and specific activities (picomoles of cAMP/min/mg of protein) calculated from linear portions of time courses are as follows: none ( $\bullet$ ), 10.0; norepinephrine ( $\circ$ ), 5.9; NaF ( $\blacksquare$ ), 34.2; NaF plus norepinephrine ( $\square$ ), 30.4; Gpp(NH)p ( $\blacktriangle$ ), 28.1; and Gpp(NH)p plus norepinephrine ( $\triangle$ ), 28.1.

phine are similar and exceed that of carbachol alone. The extent of inhibition by norepinephrine or morphine alone (about 46%) was similar to that obtained with combinations of two or three ligands. To show that the three ligands employed activate different species of receptors, each ligand was tested in the presence of dihydroergotamine, naloxone, or atropine to block  $\alpha$  receptors, opiate receptors, or muscarinic receptors, respectively. Inhibition of adenylate cyclase by each receptor activator was prevented only by the antagonist specific for the appropriate receptor.

It should be noted that the maximum inhibitions of PGE<sub>1</sub>-stimulated cAMP accumulation in intact NG108-15 cells by norepinephrine, morphine, or acetylcholine alone are in the range 75 to 90% (Refs. 19, 22, and 23, Table I), considerably greater than the maximum inhibitions of adenylate cyclase found in homogenates. These results suggest that at least three or four species of receptors can be functionally coupled to the same population of adenylate cyclase molecules or molecules regulating the enzyme.

#### DISCUSSION

The results demonstrate that the adrenergic receptors of NG108-15 cells exhibit the specificity for ligands characteristic of  $\alpha$  receptors. Activation of the  $\alpha$  receptors results in inhibition of basal and PGE<sub>1</sub>-stimulated adenylate cyclase. Inhibition by norepinephrine is dependent on GTP and is partially or completely abolished by NaF or Gpp(NH)p. The results suggest that inhibitory  $\alpha$  receptors and stimulatory PGE<sub>1</sub> receptors interact with the same population of adenylate cyclase molecules because the decrease in cAMP formed due to norepinephrine is greater in the presence of PGE<sub>1</sub> than in the absence of PGE<sub>1</sub>. Thus, norepinephrine can regulate basal adenylate cyclase activity and the sensitivity and magnitude of enzyme responses to a ligand for another species of receptor that activates the enzyme. The response of the enzyme to an activating ligand may be shifted reversibly from a subsensitive to a supersensitive state by altering the relative concentrations of activating and inhibiting ligands. In addition, the results suggest that three species of NG108-15 receptors that mediate

TABLE V

Lack of additivity of NG108-15 adenylate cyclase inhibitions mediated by  $\alpha$ , opiate, and muscarinic acetylcholine receptors

Basal adenylate cyclase activity was assayed with 116  $\mu$ g of NG108-15 homogenate protein and where indicated: 10  $\mu$ M (—) norepinephrine (NE), 10  $\mu$ M morphine, 50  $\mu$ M carbamylcholine chloride (carbachol), 10  $\mu$ M dihydroergotamine, 10  $\mu$ M naloxone, or 10  $\mu$ M atropine. The results are means of duplicate determinations.

Additions	Picomoles cAMP/min/mg protein	% of control
None	9.2	100
NE	4.9	53
Morphine	5.0	54
Carbachol	6.7	73
NE + morphine	4.7	51
Morphine + carbachol	5.0	54
NE + carbachol	5.2	56
NE + morphine + carbachol	5.2	56
Dihydroergotamine	8.4	100
NE + dihydroergotamine	7.7	92
Morphine + dihydroergotamine	5.6	67
Carbachol + dihydroergotamine	6.4	76
Naloxone	9.1	100
NE + naloxone	5.2	57
Morphine + naloxone	9.9	109
Carbachol + naloxone	7.0	77
Atropine	8.5	100
NE + atropine	5.2	61
Morphine + atropine	4.9	58
Carbachol + atropine	9.0	106
Dihydroergotamine + naloxone + atropine	9.7	100
NE + morphine + carbachol + dihydroergotamine + naloxone + atropine	8.4	87

inhibition of adenylate cyclase ( $\alpha$  receptors, muscarinic acetylcholine receptors, and opiate receptors) also interact with the same population of adenylate cyclase molecules and/or regulatory molecules because the inhibitions mediated by the three species of receptors are not additive.

At least two, and possibly more, species of  $\alpha$  receptors have been distinguished on the basis of differences in the relative potencies of ligands. It has been proposed that postsynaptic  $\alpha_1$  receptors mediate excitatory responses such as smooth muscle contraction, while presynaptic  $\alpha_2$  receptors of axons that release norepinephrine mediate the feedback inhibition of norepinephrine release at synapses and perhaps other inhibitory processes (reviewed in Refs. 33 to 35). The specificity of NG108-15  $\alpha$  receptors for ligands resembles that of the presynaptic  $\alpha_2$  receptor because the potency of  $\alpha$ -methyl-norepinephrine is high and the potencies of methoxamine and phenylephrine are low relative to that of norepinephrine; in addition, the potency of the antagonist yohimbine is high relative to that of phentolamine (Tables II and III). However, clonidine acts as a mixed agonist-antagonist with respect to NG108-15 adenylate cyclase activity, an effect characteristic of postsynaptic  $\alpha_1$  receptors (43). In addition, norepinephrine did not reduce basal or serotonin-stimulated acetylcholine release from NG108-15 cells.<sup>6</sup> These results show that the  $\alpha$  receptors of NG108-15 cells have some properties attributed to presynaptic  $\alpha_2$  receptors and at least one property attributed to postsynaptic  $\alpha_1$  receptors. Additional studies, that will be reported elsewhere, show that NG108-15 membranes con-

<sup>6</sup> S. Wilson and M. Nirenberg, unpublished results.



tain saturable sites which bind [ $^3\text{H}$ ]dihydroergocryptine with high affinity and that the  $^3\text{H}$ -ligand is displaced by ligands that are known to interact selectively with  $\alpha$  receptors.<sup>7</sup>

Human platelets, the only other cell type in which  $\alpha$  receptor-mediated inhibition of adenylate cyclase has been unequivocally demonstrated (26, 38, 44), possess  $\alpha$  receptors that resemble NG108-15 receptors, insofar as, for example, yohimbine is more potent than phentolamine. It is tempting to speculate that different subclasses of  $\alpha$  receptors may mediate different biochemical responses, and that one subclass, possibly  $\alpha_2$ , may mediate inhibition of adenylate cyclase in a variety of cell types. It would be of interest to determine whether activation of  $\alpha_2$ , muscarinic acetylcholine, or opiate receptors of presynaptic nerve terminals results in a reduction of intracellular cAMP, and if so, whether decreases in cAMP concentration are required for the inhibition of neurotransmitter release elicited by ligands interacting with these receptors (reviewed in Ref. 34).

Micromolar concentrations of GTP are required for  $\alpha$  receptor-mediated inhibition of NG108-15 adenylate cyclase. GTP stimulates the initial rate of adenylate cyclase activity, and norepinephrine inhibits the GTP-dependent increase in enzyme activity. However, the GTP concentration resulting in maximal stimulation of adenylate cyclase activity (0.2  $\mu\text{M}$ ) is less than that required for maximal inhibition by norepinephrine (1  $\mu\text{M}$ ). Higher concentrations of GTP are inhibitory. These results suggest that GTP has multiple effects on basal adenylate cyclase activity. A GTP requirement for  $\alpha$  receptor-mediated inhibition of human platelet adenylate cyclase has been reported (45), and a similar GTP requirement for opiate receptor-mediated inhibition of NG108-15 adenylate cyclase by morphine has been found.<sup>8</sup> The demonstration that GTP is required for receptor-mediated inhibition of adenylate cyclase must be considered in the context of models that attempt to describe the role of GTP in receptor-mediated activation of adenylate cyclase (46-49). We suggest that GTP is required for coupling of receptors to adenylate cyclase and that this process may result in either activation or inhibition of the enzyme, depending on the species of receptor.

In a number of cell types, an increase in intracellular cGMP accompanies a reduction of the cAMP level (50).  $\alpha$  Receptor activation elevates the cGMP level in some tissues (13, 14) but has no effect on the cGMP level of NG108-15 cells (Table I). Thus,  $\alpha$  receptor-mediated decreases in the cAMP level are not necessarily associated with alterations in the cGMP level.

Clonal NG108-15 cells are relatively homogeneous and easily obtained in large quantities. These cells generate action potentials and synthesize, store, and secrete acetylcholine and form synapses with striated muscle cells. The cells, therefore, can be used as a model system for studies on  $\alpha$  receptor properties and possible functions, including those that may regulate trans-synaptic communication. In the accompanying report (24), we show that  $\alpha$  receptors of NG108-15 cells mediate long term as well as short term effects on adenylate cyclase activity.

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